The effect of charge-reversal amphiphile spacer composition on DNA and siRNA delivery

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Abstract

A series of charge-reversal amphiphiles with different spacers separating the head group from the hydrophobic chains are described for delivery of DNA and siRNA. Among them, the amphiphiles possessing a glycine spacer (e.g., **B-GlyGly**) showed effective DNA transfection in CHO and NIH 3T3 cells as well as siRNA gene knockdown in HepG2 and UASMC cells. Ethidium bromide quenching assays revealed that DNA was released the fastest from the lipoplex of **B-GlyGly** in the presence of esterase. Also, X-ray diffraction results indicated that the DNA was located between the adjacent lipid bilayers in the lipoplex of **B-GlyGly**. These distinct features appear to be required for high transfection activity.

Introduction

Gene therapy has received significant attention in the past decade due to its advantages over traditional therapies. The two most common methods for gene delivery use either synthetic (1-5) or viral(6-10) vectors. Compared to viral vectors, non-viral vectors have lower toxicity, high load capacity and are easy to synthesize. Consequently, there has been a significant effort to develop and evaluate non-viral vectors, which include cationic amphiphiles and polymers(11-15) and more recently anionic amphiphiles.(16) Yet, synthetic vectors also have a number of limitations including low transfection efficiencies both *in vitro* and *in vivo*, significant cytotoxicity, and inactivation in the presence of serum. To overcome these limitations, investigators are modifying the amphiphile structure and, for small cationic amphiphiles, this entails alternative cationic head groups, linkers, and hydrophobic moieties. Examples of different cationic head groups explored include choline, (17,18) spermine,(19) di- and tri-peptides,(20,21) carbohydrates,(22,23) and nucleosides. (24-32) With regards to the hydrophobic moiety, long chain ester and ether linked hydrocarbons have been extensively studied. The fluorinated analogues of cationic lipids, like DOTMA, have also been explored and these amphiphiles exhibited higher transfection activities *in vitro* (33) and *in vivo* compared to their hydrocarbon analogs.(34) In addition, researchers have used combinatorial approaches to screen large libraries of amphiphiles in order to identify key structural components of the amphiphile responsible for high transfection activity.(35,36)

Although significant research has been conducted regarding the structure-property relationships of a wide range of cationic lipids, only a few studies can be found that systematically investigate the effect of spacers within the head group, and include cationic lipids derived from betaine glycine,(37) cationic glycolipids,(38) and cholesterol-based cationic lipids.(39) These studies, along with others, have begun to provide design parameters for optimizing the chemical structure for efficient nucleic acid delivery with minimal cytotoxicity.

In previous research in our lab, we found that a charge-reversal amphiphile (Figure 1A) which possesses benzyl esters at the end of alkyl chains showed high transfection activity. (40) The total charge of this amphiphile was capable of switching from +1 to -1 when the benzyl esters are hydrolyzed. This charge-reversal effect was implemented to facilitate the release of DNA from the amphiphiles. In a separate study, we also found that spacing of the cationic charges within the headgroup in a series of peptide-based amphiphiles can afford improved transfection performance with amphiphiles **KWK-C14**, **KGK-C14** and **KGG-C14** showing higher transfection compared to **KK-C14** (Figure 1B).(21) To take advantage of the charge-reversal effect, as well as to further understand how spacer length and composition affect transfection efficacy, here we present a study on a series of charge-reversal amphiphiles with different spacers separating the head group from the hydrophobic chains.

All the amphiphiles studied (Figure 2) possess a quaternary ammonium as a cationic head group (**B**) and the same alkyl chains ending with benzyl esters, but have spacers of different lengths: two, five or seven atoms. The nature of the spacers is also varied: 1) the glycine and alaine spacers are relatively rigid, hydrophilic and capable of hydrogen bonding (**B-Gly**, **B-GlyGly**, **B-AlaAla**; 2) the ethylene oxide spacers are flexible and hydrophilic (**B-EO**, **B-(EO)$_2$**); and 3) the alkyl chain spacers are flexible and hydrophobic (**B-(CH$_2$)$_4$**, **B-(CH$_2$)$_7$**).

Results and Discussion

The synthetic routes to the different amphiphiles can be found in Scheme 1 and a detailed procedure can be found in the SI. In general, the different spacers containing the head group were first synthesized and then coupled to **1** or **2** to afford the desired products. As a representative example, the synthesis of the **B-GlyGly** amphiphile is described next. N,N-dimethylglycine hydrochloride and glycine benzyl ester hydrochloride were reacted in the presence of DCC and DMAP at RT for 16h. Benzyl 2-(2-(dimethylamino)acetamido)acetate was purified as yellow oil (76%). The benzyl group was removed in the presence of a catalytic amount of Pd(II) on carbon at RT under 50psi H$_2$ for 16h to afford the intermediate, in 84% yield. The deprotected 2-(2-(dimethylamino)acetamido)acetic acid was then coupled with **2** followed by reaction with CH$_3$I to afford the **B-GlyGly** amphiphile as an viscous oil in 80% yield. The characterization data for **B-GlyGly,** as well as all of the amphiphiles, can be found in the SI.

To determine whether the amphiphiles would bind DNA, a standard ethidium bromide (EtBr)-DNA fluorescence quenching assay was performed, as shown in Figure 3 (top). DNA binding was observed for all the amphiphiles with similar affinity. Among the amphiphiles, **B-(CH$_2$)$_4$** and **B-(CH$_2$)$_7$** showed the strongest binding affinity with a +/- charge ratio of 6:1, and **B** bound the weakest with a charge ratio of 9:1. Next, the above DNA/EtBr/amphiphile solution was incubated with esterase at pH 7.4 (100mM Tris, 100mM NaCl buffer, 300 units/mL). The intensity of fluorescence, which reflects the dissociation of the amphiphile from the DNA as a consequence of the hydrolysis of the terminal benzyl esters, increased at slightly different rates for the amphiphiles, as shown in Figure 3 (bottom). DNA was released the fastest from the **B-GlyGly** complex with around 30% fluorescence recovered in

the first 30 minutes, while less than 10% fluorescence recovery was observed for all the other complexes. During the 4 hour period, fluorescence intensity increased slowly and reached ~20-35% for all other complexes, except for **B** where the fluorescence did not change significantly.

Given their polar headgroup and long hydrophobic alkyl chains, these amphiphiles are likely to form bilayer vesicles in aqueous solution. Differential scanning calorimetry (DSC) traces of hydrated amphiphiles showed phase-transition temperatures between ≈ -22 and -15 °C, except for **B** which was 60.6°C (Table 1). We subsequently prepared liposome solutions of all the amphiphiles by sonicating a hydrated film of the amphiphile in an aqueous solution (see SI for details). Dynamic light scattering measurements revealed that the sizes of liposomes in the presence of DNA varied from 154 to 353 nm in pH 7.4 solution (100 mM NaCl, 100 mM Tris buffer). The stability of the liposomes over time was examined by measuring their sizes at 0, 1, 2 and 4 h (Figure 4 top). The size of liposomes formed from **B-Gly, B-(EO)$_2$** and **B-(CH$_2$)$_7$** remained almost constant during the 4 h period, while those of other amphiphiles increased moderately. Given that these increasing rates are very small, all the liposomes appeared to be stable.

Since endocytosis is one of the major cell uptake pathways for internalization of nonviral vectors,(41-44) liposomes were incubated in a solution (100 mM NaCl, 100 mM phosphate buffer) of pH 5, which resembles the pH of the endosome, and analyzed at 0, 1, 2, 4 h (Figure 4 bottom). Generally, the size of liposomes at pH 5 is slightly smaller than those at pH 7.4. During the 4 hour period, all the liposomes were stable except for the liposome formed with **B-EO**, which quickly increased in size. The size increased from 264 to 468 nm in 1 h, and further to 655 and 1428 nm in 2 and 4 h. These findings demonstrated that the liposomes formed from the amphiphiles were stable under pH 7.4 and mildly acidic conditions, except those formed from **B-EO**.

Next, to characterize the supramolecular structures, X-ray diffraction experiments were performed at 22°C on all of the samples in the absence and presence of DNA. A hydrated vesicle pellet was obtained for all samples and oriented multilayers were prepared for X-ray analysis by placing the pellets on a curved glass substrate and incubating at 66% relative humidity. All of the samples showed multiple reflections indexing as orders of lamellar repeat periods, characteristic of lipid bilayers (Table 1).

The repeat periods of all samples in the absence of DNA were between 5.4 and 7.6 nm, except for **B-(CH$_2$)$_7$** which had a period of 1.4 nm. This period was abnormally small and could be a higher order reflection from a larger repeat period. The addition of DNA increased the repeat period for all of the assemblies. A smaller increase between 0.1 and 0.6 nm was observed for **B**, **B-Gly**, **B-(CH$_2$)$_4$**, **B-(EO)$_2$**, and **B-AlaAla**. While, for **B-EO** and **B-GlyGly**, we observed an increase in the period of 1.0 & 1.8 nm, respectively. This larger change would be consistent with a structural model where a smectic phase was formed with the DNA chains located between the adjacent lipid bilayers within the multilamellar liposome. This structural model has been reported for complexes of DNA with DOTAP(45) and cationictriesters of phosphatidylcholine.(46) For the other samples, the repeat periods did not change significantly, indicating that DNA was not located within the lipid multilayers.

Transfection experiments using reporter gene, β-galactosidase (β-gal, pVax-LacZ1, Invitrogen) were performed with CHO and NIH 3T3 cells (Figure 5). Gene transfection results were determined after 48 h as a function of cation/anion ratios (4:1, 8:1, 12:1, 16:1).

In CHO cells, amphiphiles **B-Gly** and **B-GlyGly** showed the highest transfection activity, amphiphile B-AlaAla exhibited moderate activity, and the other amphiphiles showed

minimal transfection efficiency. **B-GlyGly** was significantly more active than the positive control, Lipofectamine™ 2000. However, both the amphiphiles **B-Gly** and **B-GlyGly** were less active than Lipofectamine™ 2000 in NIH 3T3 cells, but still had higher transfection efficiency compared to the other amphiphiles we tested. These results showed that those amphiphiles containing spacers that are hydrophilic, relatively rigid, and capable of hydrogen-bonding possessed the highest transfection activity. We also prepared the **B-AlaAla** amphiphile since this amphiphile is similar to the **B-GlyGly** amphiphile but the spacer is slightly more hydrophobic and rigid. The **B-AlaAla** amphiphile showed moderate transfection activity but less than that observed with the **B-GlyGly** amphiphile. To confirm that the benzyl-ester linkages on the hydrophobic chains (and the resulting charge-reversal effect) was a contributor to the gene delivery efficiency of these amphiphiles, we synthesized two additional control amphiphiles with the Gly or GlyGly spacers, possessing saturated C16 chains instead of the benzyl ester terminated hydrophobic chains. As shown in Figure 6, both amphiphiles displayed minimum transfection activity in CHO cells. This result demonstrated the importance of charge-reversal effect on the transfection. MTS assays performed with all the amphiphiles showed no significant cytotoxicity in the two cell lines (see SI).

Next, siRNA transfections were performed with the base amphiphiles **B** and the two best performing amphiphiles, **B-Gly** and **B-GlyGly**, using the KDalert™ GAPDH assay (Ambion) with HepG2 and UASMC cells (Figure 7) at four different cation/anion ratios (1:1, 5:1, 10:1, 15:1). NeoFX was used as the positive control. After 48 h transfection, in HepG2 cells, **B** and **B-Gly** achieved 50% gene knockdown, while **B-GlyGly** only achieved 20% knockdown. In UASMC cells, **B** and **B-Gly** achieved 50% gene knockdown, while **B-GlyGly** reached 60% knockdown. These results were comparable to those obtained with NeoFX.

Conclusion

In summary, a series of amphiphiles with different spacers within the head group were evaluated for DNA and siRNA delivery. Among them, the amphiphiles possessing the relatively rigid and hydrophilic glycine or alaine spacers, capable of hydrogen bonding, showed effective DNA transfection *in vitro*. As for siRNA transfection, **B**, **B-Gly** and **B-GlyGly** showed gene knockdown. The binding affinity for the amphiphiles with the peptide spacers was similar to all the other amphiphiles, but DNA was released the fastest from **B-GlyGly** in the presence of esterase. Also, X-ray diffraction results indicate that DNA is located between the adjacent lipid bilayers in the complex formed with **B-GlyGly**. It is clear that many factors can influence transfection efficiency and that small perturbations in structure can have a significant effect. In this study, we found that the best performing amphiphile had a peptide linker separating the head group from the chains, complexes with DNA to afford a bilayer structure, and forms lipoplexes of 200 nm in diameter. This study highlights the importance and sensitivity of the spacer group in DNA/amphiphile assembly, liposome stability, and gene transfection activity. Continued studies with different amphiphile compositions will facilitate the development of a set of design requirements for efficient DNA and RNA delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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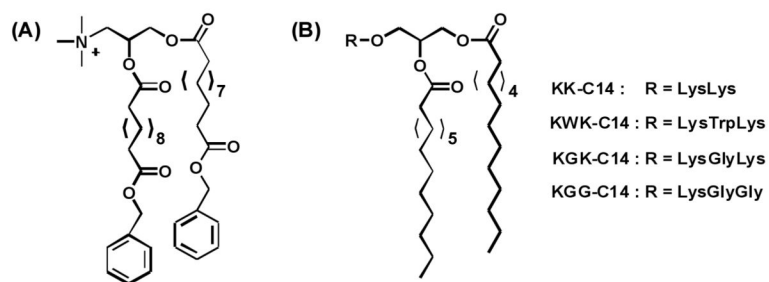
Figure 1.
Amphiphiles previously studied.
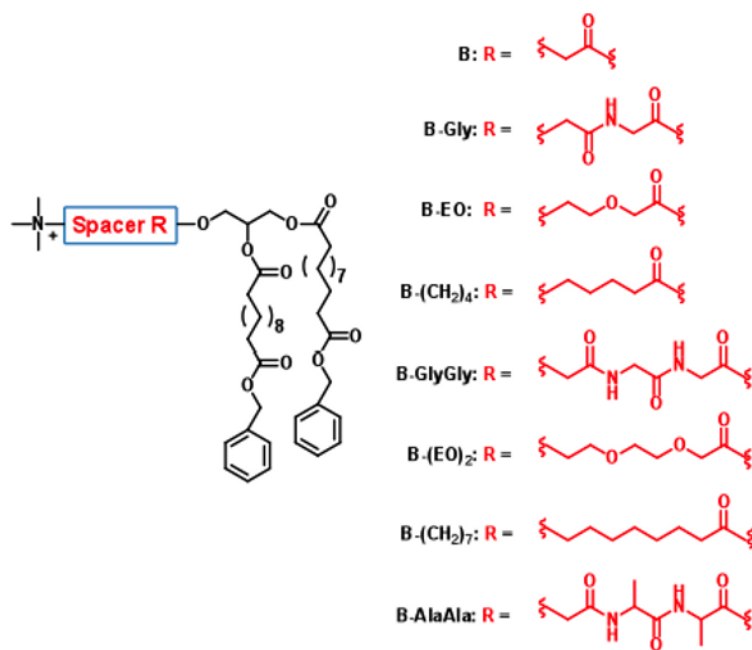
Figure 2.
Amphiphiles with different spacers.
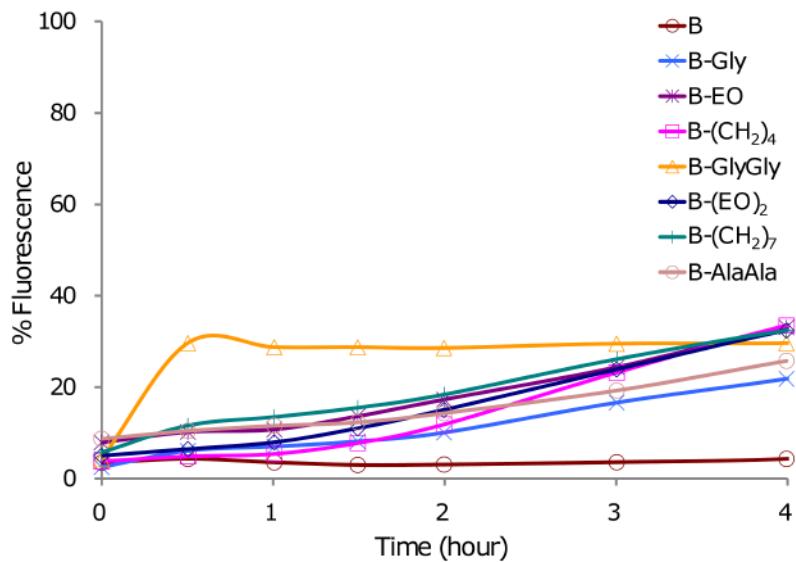
Figure 3.
EtBr displacement assay showing the fluorescence intensity. Top: as a function of amphiphile/DNA charge ratio; bottom: as a function of time in presence of porcine liver esterase (300 units/mL).
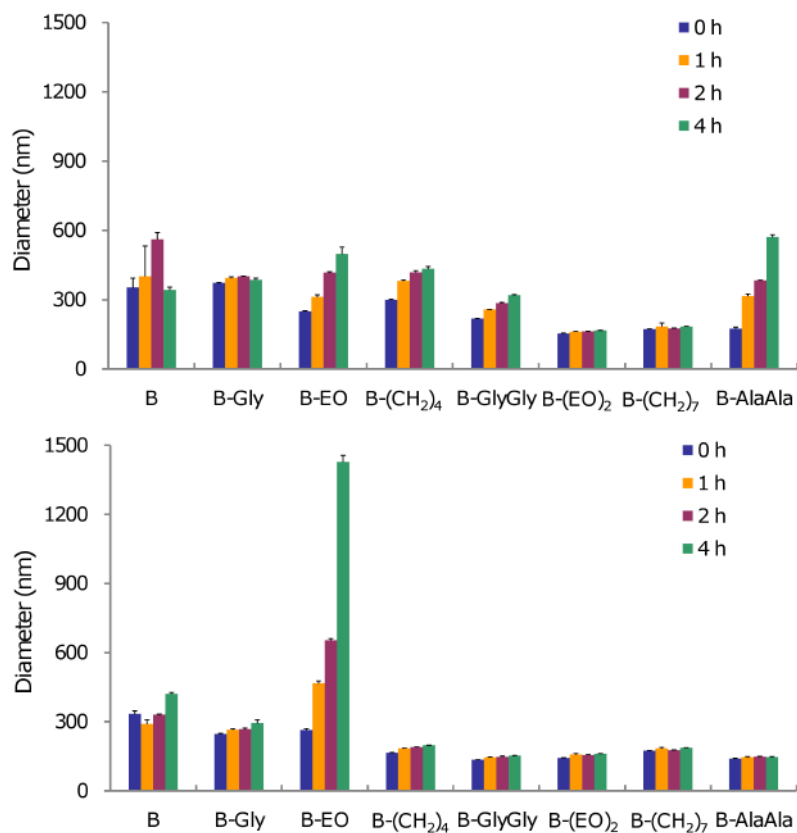
Figure 4.
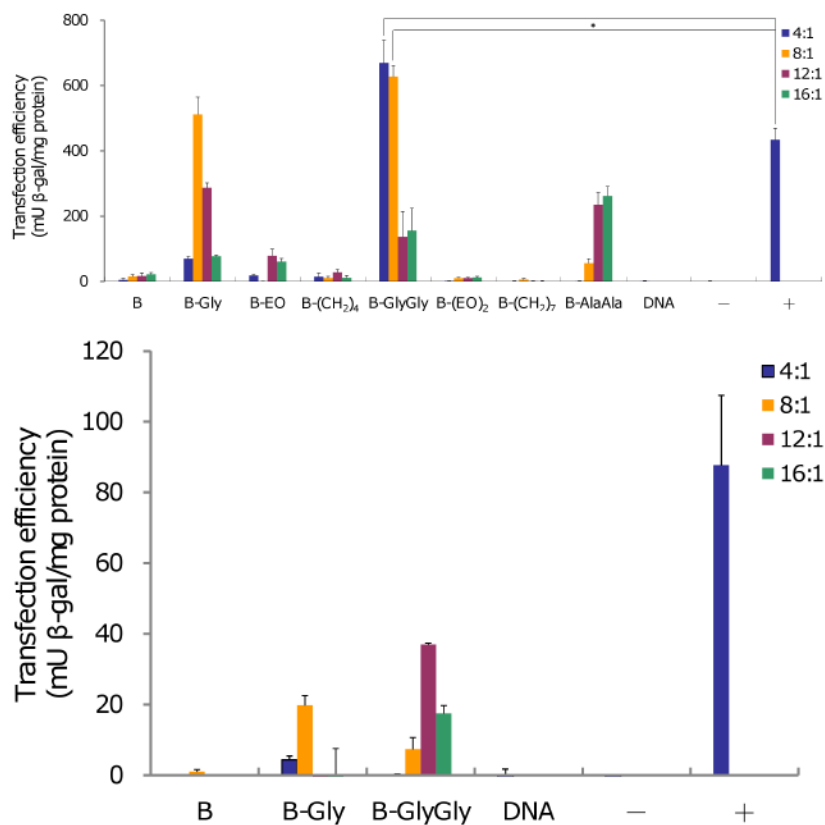Sizes of lipoplexes (nm) at pH 7.4 (top) and pH 5 (bottom). N=3, mean±SD

Figure 5.
DNA transfection after 48 h in CHO (top) and NIH3T3 (bottom) cells as a function of amphiphiles and DNA molar ratio. Lipofectamine™ 2000 was used as positive control. N=3, mean±SD. * p<0.05
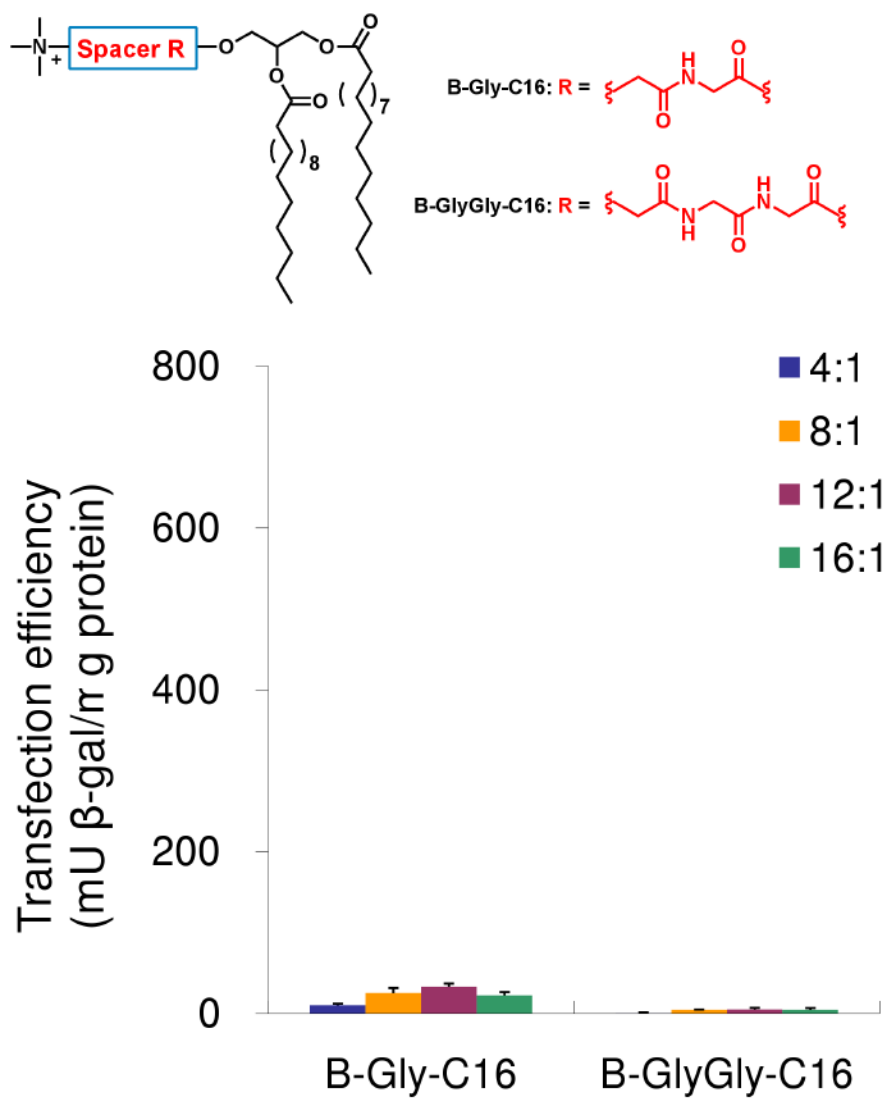
Figure 6.
Structures of **B-Gly**-C16 and **B-GlyGly**-C16 and DNA transfection after 48 h in CHO cells as a function of amphiphiles and DNA molar ratio. Lipofectamine™ 2000 was used as positive control. N=3, mean±SD
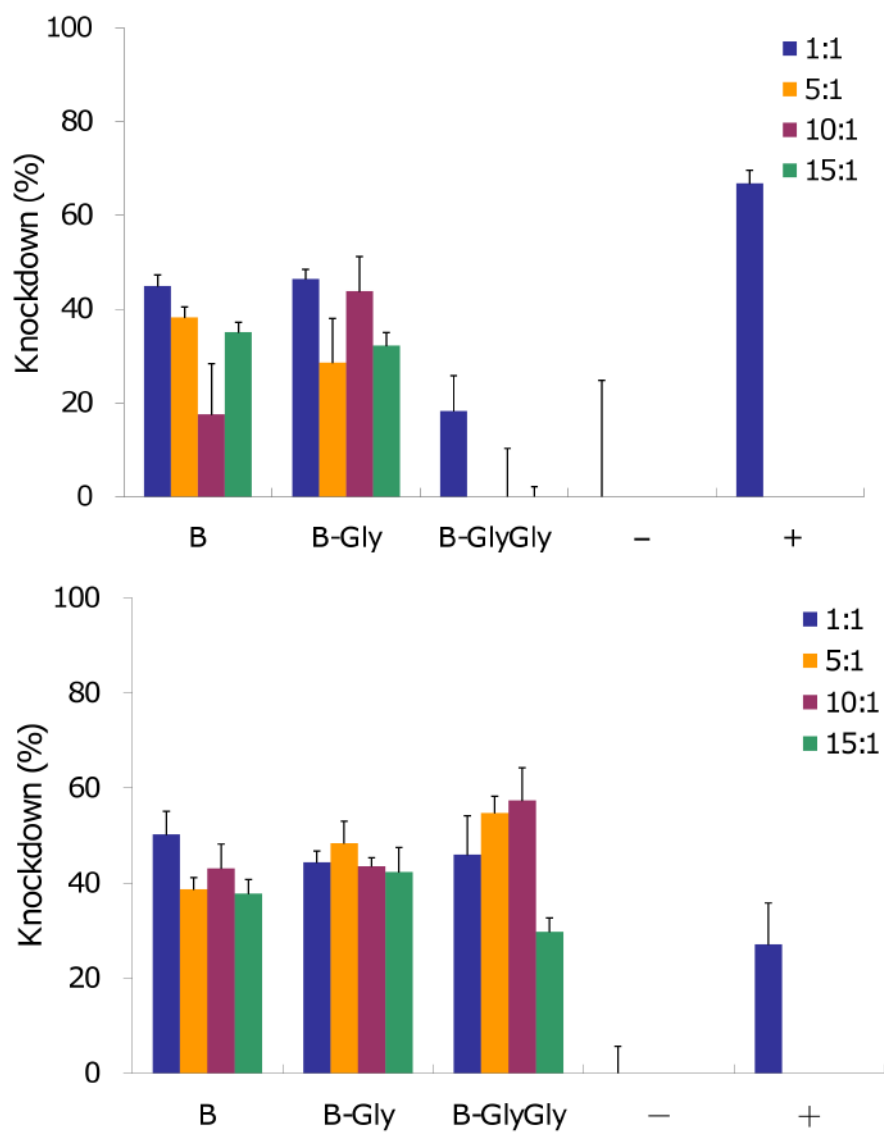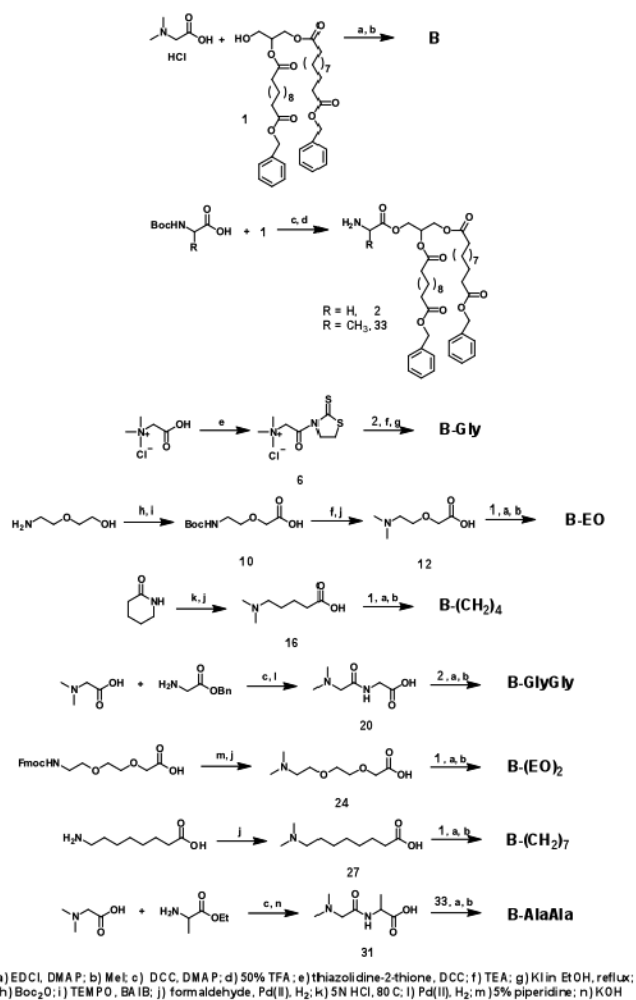
Figure 7.
siRNA transfection after 48 h in HepG2 cells (top) and UASMC cells (bottom) as a function of amphiphiles and siRNA molar ratio, NeoFX™ was used as positive control. N=3, mean ±SD

Scheme 1.
Synthesis of the cationic amphiphiles.

Table 1

Phase-transition temperatures of the amphiphiles and repeat periods without and with DNA.

Amphiphiles	Tm (°C)	Repeating period (nm)	
		without DNA	with DNA
B	60.6	5.9	6.2
B-Gly	-15.4	5.4	5.6
B-EO	-22.2	5.7	6.7
B-(CH$_2$)$_4$	-17.3	7.6	7.8
B-GlyGly	-16.8	6.1	7.9
B-(EO)$_2$	-19.6	6.4	6.5
B-(CH$_2$)$_7$	-14.8	1.4	5.8
B-AlaAla	-19.3	6.4	7.0